Determination of Nuclease Activity

The present invention is concerned with a method and kit for detecting the presence of a nuclease enzyme.

Self (EP0027036A, EP0049606A, EP0058539A, EP0060123A, US4446231, US4595655, US4598042, and US4769321) discloses methods for detecting phosphatase enzymes that produce NAD or NADH from NADP or NADPH respectively.

Akihiro (US5589349) discloses the use of enzymes with improved stability in a cycling assay for alkaline phosphatase.

Fisher et al disclose the assay of nucleases using FADP as a substrate (WO98/19168A).

Harbron et al (Analytical Biochemistry (1991) **198**:47-51) disclose an assay for alkaline phosphatase which relies on the production of FMN, which is detected using apoglycolate oxidase.

Harbron et al (Journal of Bioluminescence and Chemiluminescence (1991) **6**:251-258) disclose the luminometric detection of alkaline phosphatase based on the production of FMN, which is detected using the bacterial bioluminescent system.

Harbron (GB2324370B) discloses the use of nuclease P1 in a nucleic acid hybridisation assay in which excess probe is destroyed.

Stanley (Methods in Enzymology (1978) **57**:215-223) discloses the quantitation of NADH, NADPH and FMN using bacterial luciferase.

Rabin et al. (US4745054) discloses prosthetogenic enzyme amplification assays in which a pyrimidine ribonucleoside 3′-phosphate ester RpX is hydrolysed by ribonuclease to give XOH. XOH is a prosthetic group or a prosthetic group precursor such as thiamine, riboflavin, pyridoxal or pyridoxamine.

A number of patents assigned to Tropix describe 1,2-dioxetane derivatives of utility in chemiluminometric detection (US5869705, US5869699, US5866389, US5856522, US5851771, US5847161, US5840919, US5783381, US5777133, US5763681, US5756770, US5707559, US5679803, US5679802, US5652345, US5639907, US5637747, US5625077, US5605795, US5538847, US5397852, US5342966, US5330900, US5326882, US5225584, US5220005, US4978614, US4956477, US4931569, US4931223, US5843681, US5831102, US5773628, US5591591, US5582980, US5543295, US5145772, and US4952707).

The above citations are included herein by reference in their entirety.

EP-A-156641 discloses a method for detecting a ribonuclease enzyme label which hydrolyses compounds of the formula RpX to give Rp and X.

Anal.Biochemistry, 96/2, 464-468, 1979 discloses a method for the detection of RNase comprising the use of uridylyl(3'-5')adenosine, UpA, as substrate and the detection of A.

BBA 289(2), 323-330 (1972) discloses the use of adenosine-3'-α-naphthylphosphate for the fluorometric detection of ribonuclease.

Broadly, the present invention discloses in a first aspect a method for detecting binding events between specific binding pairs in which one of the pair is labelled with a nuclease enzyme, wherein the nuclease enzyme is selected from the group of nuclease enzymes that cleave a compound of formula RpX to yield R and pX and whereby the nuclease enzyme label is detected by the steps of:

a) contacting the nuclease enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is an esterifiable moiety or, only if R is a 3' nicotinamide derivative, X is an esterifiable moiety or H, whereby ROH and pX are produced, and

b) detecting said pX moiety or, only if R is a 3' nicotinamide derivative, detecting the pX moiety or the ROH moiety.

In one preferred embodiment, the present invention discloses a method for detecting binding events between specific binding pairs in which one of the pair is labelled with a nuclease enzyme, wherein the nuclease enzyme is selected from the group of nuclease enzymes that cleave a compound of formula RpX to yield R and pX and whereby the nuclease enzyme is detected by contacting said nuclease enzyme with a compound of formula RpX, wherein R is a 3'nicotinamide derivative, p is a phospho radical, and X is H or an esterifiable moiety, whereby ROH and pX are produced, and detecting said ROH moiety.

The method of the invention may be applied as a detection step in nucleic acid hybridisation assays, enzyme immunoassays and ligand:receptor binding assays

In preferred embodiments the invention provides a variety of methods for detecting the detectable moieties produced. These include fluorometric, colorimetric, and luminometric endpoints. Enzyme cycling and apoenzyme reactivation assays are also provided.

In a further aspect the invention provides a kit for carrying out the method.

Preferred embodiments of the invention may enable one to achieve one or more of the following objects and advantages:

(a) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields products that are easily detected. An advantage of the present invention is that the assay may be easily performed using equipment commonly available in the laboratory.

(b) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields a product that is a prosthetic groups for an enzyme. An advantage of the present invention is that the assay is rapid and/or has high sensitivity.

(c) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields a product that can be detected by chemiluminescent or bioluminescent means. An advantage of the present invention is that the assay is rapid and/or has high sensitivity.

(d) to provide a method to detect a complex formed between two members of a specific binding pair, in which one of said members is labelled with a nuclease enzyme. An advantage of the present invention is that the complex may be rapidly and/or sensitively detected.

(e) to provide a kit for carrying out the method of the invention.

Some embodiments of the invention will be described in more detail, by way of example, with reference to the accompanying drawings in which:

Figure 1 is a diagrammatic representation of the conversion of NAD3P to NAD through the action of a nuclease enzyme, and subsequent cycling of the NAD produced through the action of a dehyrogenase and a diaphorase enzyme, to produce a coloured formazan.

Figure 2 is a diagrammatic representation of the hydrolysis of a substrate by a nuclease enzyme to yield products for detection.

Figure 3 is a diagrammatic representation of the hydrolysis of a adenosine-3'-phosphoriboflavin derivative by the action of a nuclease enzyme to yield FMN, and subsequent reconstitution of an apoenzyme by the FMN to yield holoenzyme for detection.

Figure 4 is a diagrammatic representation of the hydrolysis of a nucleoside-3'-phospho-1,2-dioxetane derivative to yield the corresponding 1,2-dioxetane phosphate. This latter is converted to 1,2-dioxetane, which decomposes producing light.

Figure 5 is a standard curve for the detection of nuclease P1 in a NAD-NADH cycling reaction. The absorbance produced after 800 sec at different pH values is plotted against the amount of nuclease P1 present in the reaction mixture.

Figure 6 is a standard curve for the detection of FMN in an apoenzyme reconstitution assay. The absorbance produced at 324 nm is plotted against the concentration of FMN in an aliquot added to the reaction mixture for two different apoenzyme preparations: circle is sugar beet, triangle is spinach.

The present invention provides a method for detecting a nuclease enzyme.

The present invention provides a variety of methods for detecting ROH or pX. These approaches may be colorimetric, fluorimetric, or luminometric, or may be through enzyme cycling reactions or apoenzyme reactivation assays.

In a preferred embodiment, the substrate RpX is NAD3'phosphate (NAD3P). This differs from commonly occurring NADP, which carries the phosphate moiety at the 2' position. This is hydrolysed by a nuclease to give NAD, which may be easily and sensitively detected, either by spectrophotometry or fluorimetry, or by coupling with a bacterial luminescence system to produce light, as disclosed by Stanley, or through enzyme cycling, as disclosed by Self. In a preferred embodiment, NAD is converted to NADH through the action of a dehydrogenase enzyme. The dehydrogenase enzyme may be alcohol dehydrogenase or lactate dehydrogenase. The presence of NADH may be detected spectrophotometrically or fluorometrically. Referring to Fig. 1, which shows a particularly preferred embodiment, NAD3P is hydrolysed to give NAD. NAD is converted to NADH through the action of a dehydrogenase enzyme. A diaphorase enzyme reduces a tetrazolium compound, such as INT, to give NAD and a coloured formazan, the absorption of which can be measured at 492nm. The NAD produced may then be cycled back to NADH through the action of the dehydrogenase enzyme, leading to an ever-increasing rate of colour development. A similar approach may be used using NAD3PH as the substrate, which yields NADH on hydrolysis. In this embodiment, the NADH enters the cycle as a substrate for diaphorase.

Referring now to Fig. 2, which shows another preferred embodiment, the substrate is a nucleosidyl-3'-phosphodiester wherein X is, for example, riboflavin, thiamine, pyridoxamine or pyridoxal, B is a base, for example adenine, guanine, uracil, thymine or cytosine, or a derivative thereof, and R' is H or other substituent. These are hydrolysed by the nuclease enzyme to yield, for example, riboflavin phosphate (FMN), thiamine phosphate, pyridoxamine phosphate or pyridoxal

phosphate, respectively. These may be detected using an apoenzyme reactivation assay of the type disclosed by Rabin. For example, FMN may be detected using apoglycolate oxidase; pyridoxal phosphate may be detected using apoaminoacid transferase. In a particularly preferred embodiment, shown in Fig. 3, the substrate adenosine-3'-phosphoriboflavin wherein A is adenine and R' is H. This compound is hydrolysed by the nuclease enzyme to yield adenosine and FMN (riboflavin phosphate). FMN may be sensitively detected using an apoenzyme, such as apoglycolate oxidase, as described by Harbron et al. (Analytical Biochemistry (1991) **198**:47-51), or by bioluminescent detection, as described by Harbron et al. (Journal of Bioluminescence and Chemiluminescence (1991) 6:251-258). In an analogous fashion, RpX may be adenosine-3'-phosphothiamine, adenosine-3'-phosphopyridoxamine or adenosine-3'-phosphopyridoxal, which upon hydrolysis yield thiamine phosphate, pyridoxamine phosphate or pyridoxal phosphate. These two may be sensitively detected using the corresponding; for example, glycolate oxidase, or a transaminases.

In a further preferred embodiment, illustrated in Figure 4, the substrate is a nucleosidyl-3´-phospho-1,2-dioxetane derivative, such as a nucleosidyl-3´-phosphoadamantyl derivative, wherein B is a base, for example adenine, guanine, uracil, thymine or cytosine, or a derivative thereof, and R' is H or other substituent. This is hydrolysed to yield an adamantyl phosphate derivative, which may be hydrolysed chemically or in the presence of a phosphatase enzyme to yield the corresponding adamantyl derivative, which decomposes chemiluminometrically. A further preferred embodiment utilises an adenosine-3'-phosphoadamantyl derivative, which, upon hydrolysis, yields an adamantyl-phosphate derivative. This may then be dephosphorylated by means of a phosphatase enzyme or chemically. For example, the adamantyl-phosphate derivative produced may be CDP-*Star* (R) from Tropix Inc. Upon dephosphorylation of CDP-*Star* (R) substrate by alkaline

phosphatase, a metastable chlorophenolate dioxetane anion intermediate is formed which decomposes and emits light at a maximum wavelength of 466 nm. A delay in reaching maximum light emission results since the dioxetane anion has a half-life of less than one minute to several hours, depending on the surrounding environment. Film or simple instrumentation may be used to quantitate the chemiluminescent signal, which is produced as a continuous glow due to the reaction kinetics of the system.

The nuclease enzyme is any enzyme that cleaves the substrate RpX to yield R and pX. In one embodiment the nuclease enzyme is an enzyme of class EC.3.1.30.1. In a preferred embodiment the nuclease enzyme is nuclease P1, nuclease S1 or mung bean nuclease. In a particularly preferred embodiment the nuclease enzyme is nuclease P1.

In one embodiment the nuclease enzyme is free in solution. In another embodiment the nuclease enzyme is immobilised on a surface.

The present invention provides a method for detecting binding events between specific binding pairs, in which one of the pair is labelled with the nuclease enzyme. The covalent attachment of the nuclease enzyme to this moiety is described in Fisher et al. (WO98/19168) and Harbron (GB2324370B), and may be achieved by a number of well-known methods using a wide range of heterobifunctional reagents. For example, the method of Carlsson *et al.* (*Biochem J* (1978) **173**: 723 - 737) may be used: the nuclease enzyme is reacted with 3-[(2)-pyridyldithio]propionic acid N-hydroxysuccinimide ester (SPDP) to give a 2-pyridyl disulphide-activated label. This allows disulphide exchange with a specific binding partner having a sulphydryl

group to yield a labelled specific binding partner. Other approaches for labelling the specific binding partner will be apparent to one skilled in the art.

In one embodiment the specific binding pair comprises an antibody and a hapten or antigen. In another embodiment the specific binding pair comprises a nucleic acid probe and its corresponding target sequence. In a further embodiment the specific binding pair comprises a biotin derivative and avidin, streptavidin or neutravidin. In a yet further embodiment the specific binding pair comprises a ligand and a receptor.

Thus the invention may be used to detect binding events in nucleic acid hybridisation assays, enzyme immunoassays, and receptor:ligand binding assays.

The present invention provides a kit for carrying out the method of the invention. The kit comprises a compound of formula RpX, and a detection system for detecting ROH or pX. In one embodiment, RpX is NAD3P

The following examples illustrate aspects of the invention, and are not intended to limit the scope of the invention.

EXAMPLE 1 – Assay of Nuclease P1

A premix containing the following reagents was prepared prior to the assay and stored at 4°C until required: 50l 0 .5 M citrate buffer, pH 6.3; 100l 10 m M INT; 10 l 5 mM NAD3'P; 10l e thanol; 30l d i aphorase solution (30 U/ml); 10l alcohol dehydrogenase solution (3mg/ml) and 780 l of water.

10 l aliqouts from a serially diluted solution of nuclease P1 were dispensed into the wells of a microtitre plate. 90l o f the premix were then added, and the plate incubated at room temperature. The change in absorbance at 490 nm was followed by means of a plate reader.

The performance of the assay is illustrated in Figure 5, which shows a standard curve for the assay of nuclease P1 using the above assay at pH 6.3, and at pH 6.0 and 6.7.

EXAMPLE 2 – Apoenzyme Reactivation Assay for the Detection of FMN

Apoglycolate oxidase was prepared as described by Harbron et al. (Analytical Biochemistry (1991) **198**:47-51). A standard curve for the estimation of FMN, shown in Fig. 6, was prepared as follows: 50 mM Tris-HCl buffer, pH8.3, 44 mU apoglycolate oxidase and 0.02 to 200M F M N in a total volume of 0.05 mL was incubated for 1h at room temperature. This was then added to 0.95 mL of 50 mM Tris-HCl buffer, pH8.3, containing 3.47 mM phenylhydrazine and 5.26 mM glycolic acid, and the linear rate of absorbance was measured at 324 nm.